Functional Differences between GDNF-Dependent and FGF2-Dependent Mouse Spermatogonial Stem Cell Self-Renewal

AUTHOR(S):

Takashima, Seiji; Kanatsu-Shinohara, Mito; Tanaka, Takashi; Morimoto, Hiroko; Inoue, Kimiko; Ogonuki, Narumi; Jijiwa, Mayumi; Takahashi, Masahide; Ogura, Atsuo; Shinohara, Takashi

CITATION:

Takashima, Seiji ...[et al]. Functional Differences between GDNF-Dependent and FGF2-Dependent Mouse Spermatogonial Stem Cell Self-Renewal. Stem Cell Reports 2015, 4(3): 489-502

ISSUE DATE:

2015-02

URL:

http://hdl.handle.net/2433/193679

Stem Cell Reports
Article
ISSCR
OPEN ACCESS

Functional Differences between GDNF-Dependent and FGF2-Dependent Mouse Spermatogonial Stem Cell Self-Renewal

Seiji Takashima,[1,5] Mito Kanatsu-Shinohara,[1,2] Takashi Tanaka,[1] Hiroko Morimoto,[1] Kimiko Inoue,[3] Narumi Ogonuki,[3] Mayumi Jijiwa,[4] Masahide Takahashi,[4] Atsuo Ogura,[3] and Takashi Shinohara[1,*]
[1]Department of Molecular Genetics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
[2]Japan Science and Technology Agency, PRESTO, Kyoto 606-8501, Japan
[3]The Institute for Physical and Chemical Research (RIKEN), Bioresource Center, Tsukuba 305-0074, Japan
[4]Department of Pathology, Graduate School of Medicine, Nagoya University, Nagoya 466-8550, Japan
[5]Present address: Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan
*Correspondence: tshinoha@virus.kyoto-u.ac.jp
http://dx.doi.org/10.1016/j.stemcr.2015.01.010

SUMMARY

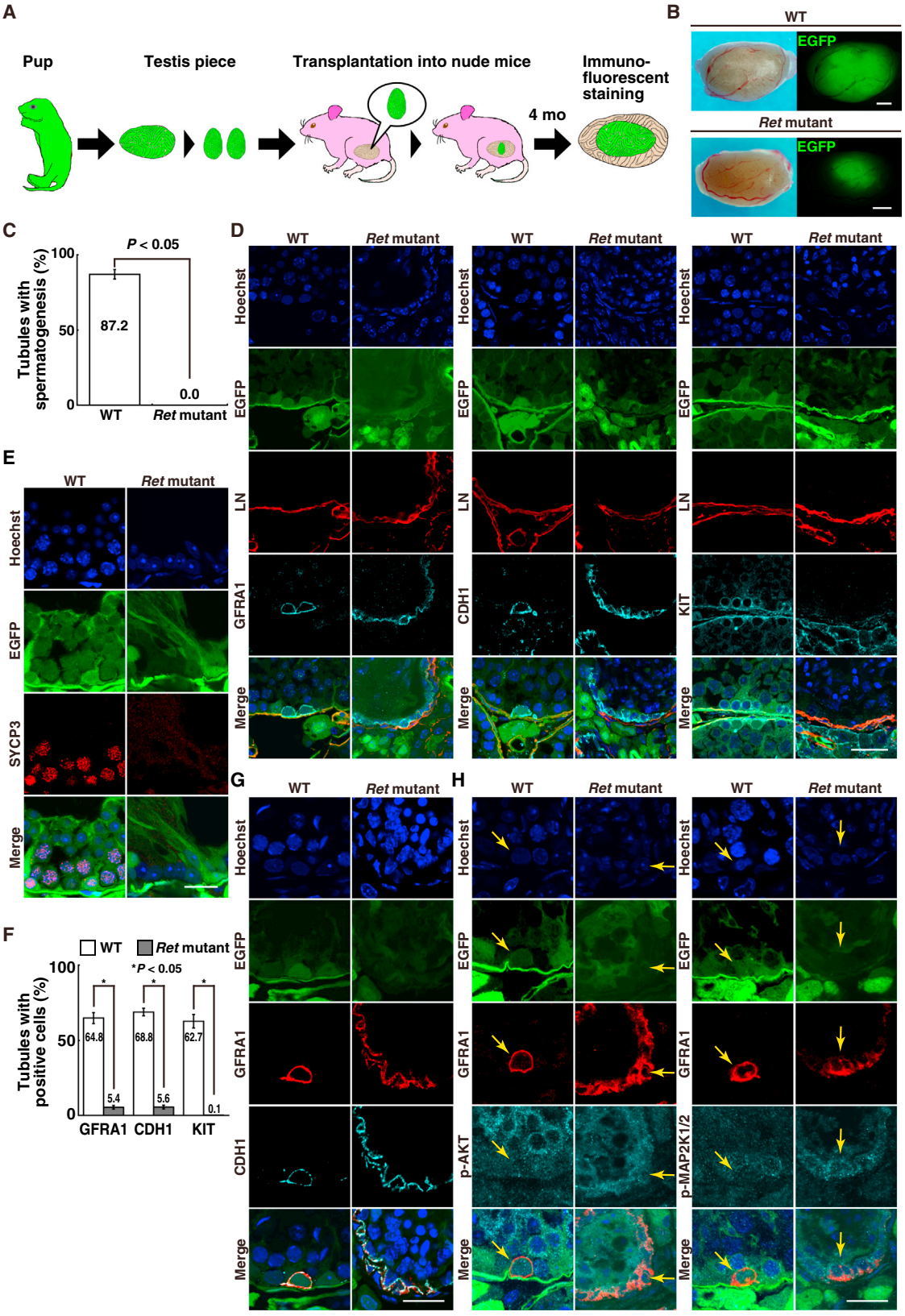
Spermatogonial stem cells (SSCs) are required for spermatogenesis. Earlier studies showed that glial cell line-derived neurotrophic factor (GDNF) was indispensable for SSC self-renewal by binding to the GFRA1/RET receptor. Mice with mutations in these molecules showed impaired spermatogenesis, which was attributed to SSC depletion. Here we show that SSCs undergo GDNF-independent self-renewal. A small number of spermatogonia formed colonies when testis fragments from a *Ret* mutant mouse strain were transplanted into heterologous recipients. Moreover, fibroblast growth factor 2 (FGF2) supplementation enabled in vitro SSC expansion without GDNF. Although GDNF-mediated self-renewal signaling required both AKT and MAP2K1/2, the latter was dispensable in FGF2-mediated self-renewal. FGF2-depleted testes exhibited increased levels of GDNF and were enriched for SSCs, suggesting that the balance between FGF2 and GDNF levels influences SSC self-renewal in vivo. Our results show that SSCs exhibit at least two modes of self-renewal and suggest complexity of SSC regulation in vivo.

INTRODUCTION

Spermatogonial stem cells (SSCs) give rise to the spermatogenesis that endures throughout the life of male animals (de Rooij and Russell, 2000; Meistrich and van Beek, 1993). SSCs reside in a special microenvironment termed a niche, which is located in the basal lamina facing the interstitium of the testis (Chiarini-Garcia et al., 2001; Yoshida et al., 2007). SSCs self-renew in this germline niche, giving rise to progenitor cells while remaining undifferentiated. Although the behavior of SSCs in the niche has been well-described in *Drosophila* (Li and Xie, 2005), little is known about SSCs of the mammalian testis, partly because the testis contains relatively few SSCs (0.02%–0.03%) (de Rooij and Russell, 2000; Meistrich and van Beek, 1993) and partly because SSCs are difficult to distinguish from committed progenitor cells via morphological analyses. Previous studies showed that SSCs produce either two stem cells after a self-renewal division or two differentiating cells after a differentiating division (de Rooij and Russell, 2000). These two types of division occur at the same frequency to maintain the SSC population at a constant level. Detailed analysis of SSCs is challenging because no SSC-specific markers are yet known. Additionally, in principle, SSCs must be defined by the ability to undergo self-renewal division, which is not easy to assess. Such problems have made it difficult to analyze SSCs and the interactions thereof within the niche described above.

In 2000, glial cell line-derived neurotrophic factor (GDNF) was shown to be involved in SSC self-renewal (Meng et al., 2000). GDNF belongs to the transforming growth factor β superfamily molecules and binds to glycosylphosphatidylinositol (GPI)-anchored GFRA1, triggering signaling via the transmembrane receptor tyrosine kinase RET, which does not directly bind to GDNF (Sariola and Saarma, 2003). In transgenic mice overexpressing *Gdnf*, undifferentiated spermatogonia accumulate in seminiferous tubules and cease differentiation. In contrast, mice heterozygous for *Gdnf* gradually lose spermatogenesis and become infertile as spermatogonia are lost (Meng et al., 2000). Knockout (KO) animals with defects in *Ret* or *Gfra1* also exhibit similar phenotypes (Jain et al., 2004; Jijiwa et al., 2008; Naughton et al., 2006). Such results suggest that SSCs undergo self-renewal when GDNF level is high, but differentiate when the GDNF concentration is low. This feature has been exploited to develop a long-term culture system for SSCs; SSC numbers increase exponentially over a 2-year period (Kanatsu-Shinohara et al., 2003). Cultured SSCs, termed germline stem (GS) cells can be subjected to gene targeting and reinitiate spermatogenesis upon transplantation into seminiferous tubules (Kanatsu-Shinohara et al., 2006). These results suggested that GDNF is a bona fide self-renewal factor for SSCs.

As GDNF plays a critical role in determining the fate of SSCs, controls on the GDNF receptor components have been investigated extensively. However, the question of



(legend on next page)

whether SSCs express such receptor components remains controversial (Buageaw et al., 2005; Ebata et al., 2005; Grisanti et al., 2009). Some authors have claimed that SSCs express GFRA1, whereas others have raised the possibility that the situation is otherwise. A transplantation assay showed that GFRA1 was transiently expressed in SSCs of immature pup testes, but not in neonate or adult SSCs (Ebata et al., 2005). Another group found that 10% of A_{single} (A_s) spermatogonia did not express GFRA1 and that 5% of A_{paired} (A_{pr}) spermatogonia asymmetrically expressed GFRA1 (Grisanti et al., 2009). Cells positive in terms of GFRA1 expression (selected using magnetic beads) were not clonogenic, whereas cells lacking GFRA1 produced colonies after transplantation. Thus, although a positive influence of GDNF in the context of SSC self-renewal has been suggested, it remains unknown why a significant proportion of A_s spermatogonia lack SSC activity and whether such spermatogonia express the GDNF receptor.

In contrast to the attention devoted to GDNF, little work has focused on exploration of the role played by fibroblast growth factor 2 (FGF2), which is thought to be essential for SSC self-renewal (Kanatsu-Shinohara and Shinohara, 2013). The effects of FGF2 have been analyzed in vitro. FGF2 induces both MAPK1/3 and AKT phosphorylation in GS cells, and cells expressing activated MAP2K1 not only induced MAPK1/3 phosphorylation but also proliferated without FGF2, albeit at a slower rate than with FGF2 and GDNF (Ishii et al., 2012). In contrast, constitutively active AKT can replace GDNF and GS cells transfected with activated AKT proliferate in the absence of GDNF (Lee et al., 2007). Studies in humans have shown that spermatogonia carrying FGF receptor mutations preferentially transmit abnormal genetic haplotypes to the next generation. Mutations in *Fgfr2* (in patients with Apert syndrome: C755G) or *Fgfr3* (in patients with Achondroplasia: G380R) are thought to occur at the SSC level because sperm mutation frequencies increase with age; mutations in progenitor cells disappear due to the lack of self-renewal activity (Bellus et al., 1995; Goriely et al., 2005). Such results suggest that hyperactivation of FGF signaling enhances SSC self-renewal; however, the relevant in vivo mechanism remains unclear.

It is generally believed that progressive loss of spermatogenesis and development of "empty" tubules, as found in *Gdnf/Ret/Gfra1* KO mice, are caused by reduced SSC self-renewal. However, we hypothesized that cessation of spermatogenesis would not necessarily indicate that SSCs were deficient. As we worked to confirm the role played by GDNF in vivo, we found that a small number of undifferentiated spermatogonia survived and formed colonies in *Ret* mutant mice, encouraging us to examine the role played by FGF2 in vivo and to seek to recapitulate SSC self-renewal in vitro in the absence of GDNF signaling.

RESULTS

Analysis of Germ Cells in Ret Mutant Mice

We examined the fate of germ cells in testis fragments of a mutant mouse strain with a point mutation in *Ret* (Y1062F). This mutation is thought to be critical in terms of SSC self-renewal (Jain et al., 2004; Jijiwa et al., 2008). We crossed such mice with a transgenic mouse line that ubiquitously expresses EGFP (green mice); the protein served as a donor marker. As such mice die within a few days of birth, testis fragments were collected from 1- to 2-day-old pups, and seminiferous tubule fragments were transplanted under the tunica albuginea of busulfan-treated nude mice (Figure 1A). Recipient testes were collected 4 months after transplantation, allowing completion of three to four cycles of spermatogenesis, thus affording sufficient time to allow of SSC depletion. Transplanted fragments from *Ret* mutant mice were generally smaller than WT fragments but could be readily identified by virtue of donor fluorescence (Figure 1B).

Although all tubules of *Ret* mutant mice were apparently empty (Figure 1C), close examination revealed that seven

Figure 1. Spermatogonial Proliferation in *Ret* Mutant Mice

(A) Experimental procedure. Mutant testes were taken from neonatal mice and were transplanted into testes of busulfan-treated nude mice. Grafts were identified by the green fluorescence of the donor transgene.

(B) Macroscopic appearance of recipient testes 4 months after transplantation.

(C) Tubules exhibiting spermatogenesis, defined by the presence of multiple layers of germ cells around the entire circumference of the tubules, were counted (n = 12 testes for *Ret* mutant; n = 4 testes for WT). At least 66 tubules were counted for each sample.

(D) Immunohistochemistry of testis samples using spermatogonial markers. LN was also stained to indicate contour of the tubule.

(E) Immunohistochemistry of SYCP3.

(F) Quantification of cells expressing spermatogonial markers. The numbers of tubules were 1,803 and 699, respectively, for *Ret* mutant and WT testes (n = 5 testes for *Ret* mutant; n = 4 testes for WT).

(G) Double immunohistochemistry of CDH1 and GFRA1.

(H) Immunohistochemical staining of $GFRA1^+$ undifferentiated spermatogonia using antibodies detecting phosphorylated AKT (p-AKT) or MAP2K1/2 (p-MAP2K1/2). Arrows indicate cells expressing p-AKT or p-MAP2K1/2.

Scale bars represent 1 mm (B), 30 μm (D and H), and 20 μm (E and G). Results are means ± SEM. See also Figure S1 and Table S3.

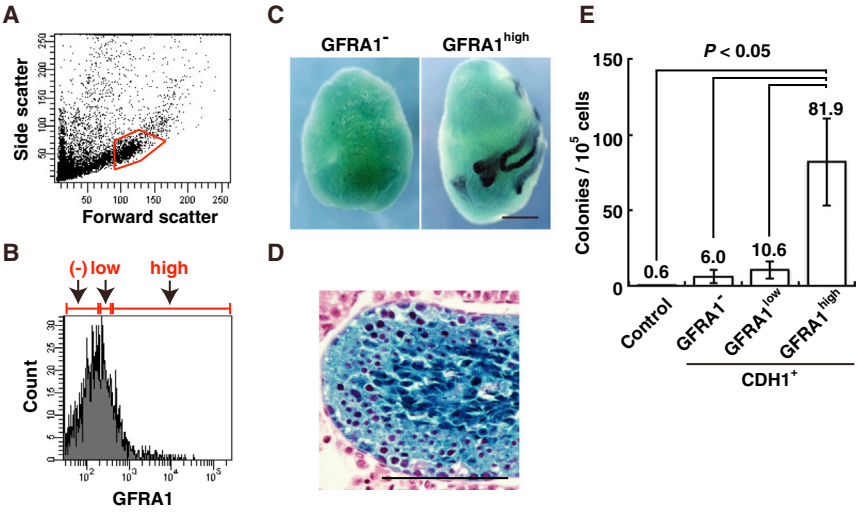


Figure 2. Enrichment of SSCs by Exploitation of GFRA1 Expression

(A) Light-scattering properties of CDH1-selected testis cells. A spermatogonial gate was set according to forward scatter (size) and side scatter (cell complexity) values.

(B) GFRA1 staining of the gated cells. Cells were sorted into three subpopulations according to the level of GFRA1 expression.

(C) Macroscopic appearance of recipient testes.

(D) Spermatogenesis in a recipient testis that was transplanted with GFRA1^{high} cells.

(E) Colony counts (n = 18–20 testes).

Scale bars represent 1 mm (C) and 100 μm (D). Results are means ± SEM. See also Figure S2 and Table S3.

of eight (87.5%) fragments contained clusters of germ cells expressing CDH1, a marker of undifferentiated spermatogonia. Staining for GFRA1, a marker of A_s, A_{pr}, and $A_{aligned}$ spermatogonia, exhibited similar patterns, but KIT, a marker of differentiating spermatogonia, was not stained (Figure 1D). In contrast, control transplants exhibited normal spermatogenesis in many tubules (Figure 1E). Importantly, the densities of CDH1- and GFRA1-expressing cells were strikingly different between mutant and WT transplants. These cells were sparsely distributed in WT testes before transplantation, and similar staining patterns were found after transplantation. However, CDH1$^+$ spermatogonia-like cells clustered densely in *Ret* mutant seminiferous tubules. Although only ~5% of all tubules contained cells expressing CDH1 or GFRA1 (Figure 1F), double immunohistochemical staining of *Ret* mutant testes showed that surviving spermatogonia-like cells usually expressed both molecules (Figure 1G), suggesting that the balance between self-renewal and differentiation was altered in *Ret* mutant mice. No apparent abnormalities in the staining pattern of laminin (LN) or collagen type I were found around the transplanted tissues, confirming that abnormal staining is not due to scar formation (Figures 1D and S1).

To explore whether the *Ret* mutation influenced downstream signaling patterns, we analyzed the phosphorylation status of AKT and MAP2K1/2, both of which are thought to be involved in SSC self-renewal. AKT and MAP2K1/2 were phosphorylated in GFRA1$^+$ spermatogonia-like cells of mutant and WT mice (Figure 1H), suggesting that these molecules were activated even in the absence of RET Y1062 signaling. Long-term survival analysis of CDH1$^+$ spermatogonia-like cells in donor cell grafts revealed that the *Ret* Y1062 mutation allowed the survival of testis cells resembling undifferentiated spermatogonia, possibly including SSCs.

Functional Analysis of GFRA1 Expression in the Testis

The results described above raised the possibility that some SSCs functioned independently of GDNF signaling, and we thus explored whether GFRA1 was expressed on SSCs. In preliminary experiments, we failed to enrich for SSCs using magnetic cell sorting (MACS) with an anti-GFRA1 antibody (data not shown). Therefore, we used fluorescent-activated cell sorting (FACS) to quantitatively evaluate GFRA1 expression levels. Testis cells from B6-TgR(ROSA26)26Sor (ROSA) mice were dissociated into single cells, and CDH1$^+$ cells, which are enriched in terms of SSCs (Tokuda et al., 2007), were collected via MACS, stained with an anti-GFRA1 antibody, and subjected to FACS (Figure 2A). The proportion of cells expressing GFRA1 in the spermatogonial gate was 56.1% ± 11.5% (n = 5), and cells were separated from CDH1-expressing cells by reference to GFRA1 expression levels (Figure 2B). Cells thus collected were transplanted into seminiferous tubules of congenitally infertile WBB6F1-W/W^v (W) mice to assess the ability of the SSCs to recolonize seminiferous tubules (Brinster and Zimmermann, 1994).

We performed three experiments, each of which featured at least nine recipient mice sacrificed 2 months after transplantation, at which time colony numbers were counted (Figures 2C and 2D). The average numbers of colonies generated by unfractionated control, CDH1$^+$GFRA1$^-$, CDH1$^+$GFRA1^{low}, and CDH1$^+$GFRA1^{high} cells were 0.6 ± 0.1, 6.0 ± 4.4, 10.6 ± 5.7, and 81.9 ± 28.8 per 10^5 cells (Figure 2E). Only CDH1$^+$GFRA1^{high} cells were significantly enriched for SSCs compared with control cells, suggesting that most SSCs expressed GFRA1. However, CDH1$^+$GFRA1$^-$ cells occasionally yielded colonies. We did not find apparent differences in distribution patterns of cells in the spermatogonia gate according to the GFRA1 expression levels (Figures S2A–S2C).

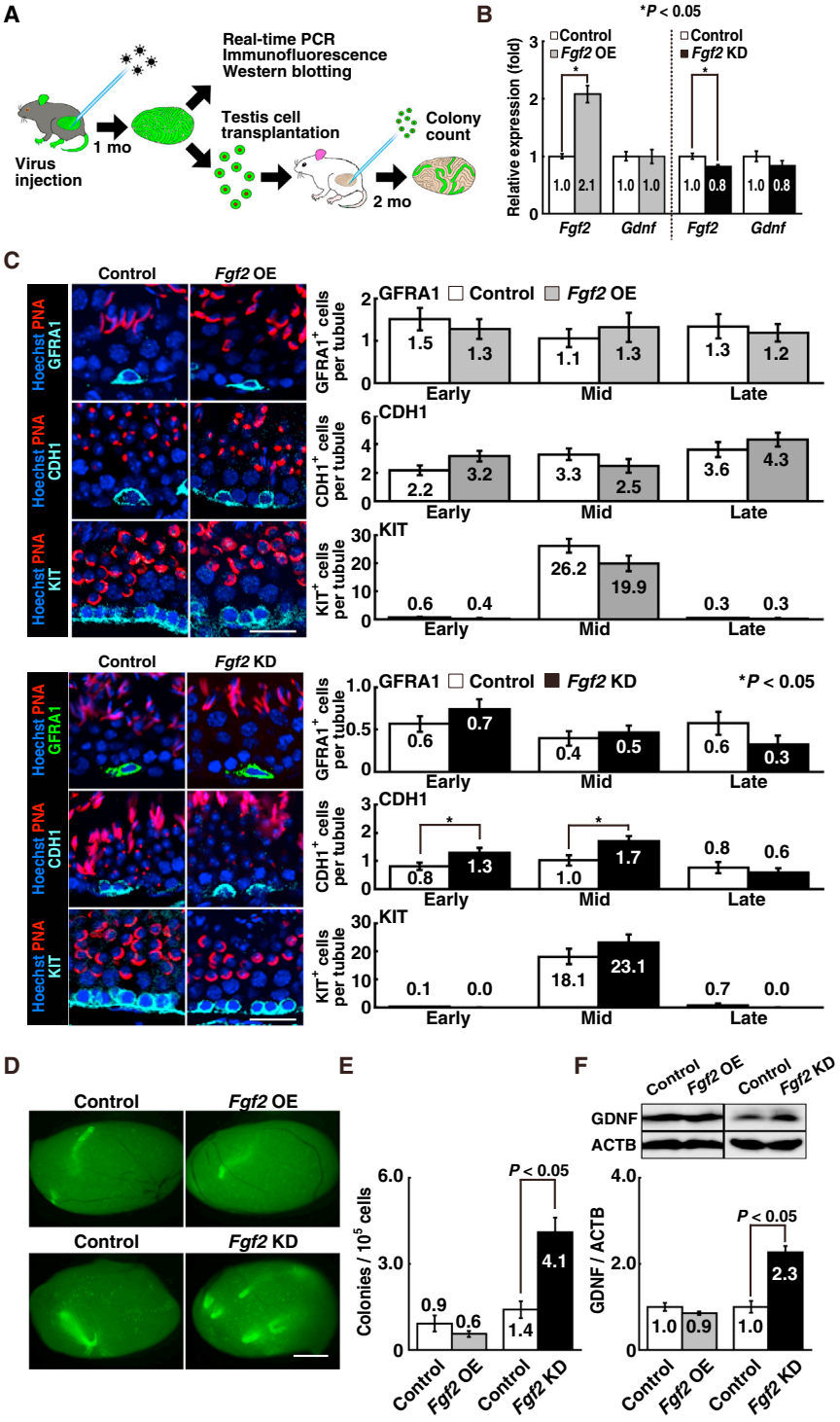


Figure 3. Effects of FGF2 Levels on SSC Activity

(A) Experimental procedure. Lentivirus particles were microinjected into seminiferous tubules of green mice. Testes were recovered 1 month after microinjection and dissociated into single cells, which were next injected into recipient mice to allow SSC activity levels to be determined.

(B) Real-time PCR analysis of testes that had received viral injections (n = 5 testes).

(C) Immunohistochemistry and quantification of spermatogonial and Sertoli cell markers in WT mouse testes subjected to *Fgf2* OE or that had received KD lentivirus (n = 3 testes). Tubules were categorized into early (I–V), middle (VI–VIII), and late (IX–XII) phases according to peanut agglutinin (PNA) staining patterns. At least 92 tubules were counted for each phase of spermatogenesis.

(D) Macroscopic appearance of recipient testes.

(E) Colony counts (n = 24 testes for overexpression [OE]; n = 18 testes for knockdown [KD]).

(F) Western blotting for GDNF and quantification of relative band intensities (n = 3–4 testes).

Scale bars represent 20 μm (C) and 1 mm (D). Results are means ± SEM. See also Figure S3 and Tables S2–S4.

Depletion of FGF2 in Seminiferous Tubules Increases SSC Activity

Based on the above observations, we hypothesized that FGF2, another critical self-renewal factor, might serve to maintain spermatogonia in *Ret* mutant mice. To explore the function of FGF2 in vivo, we performed two sets of experiments using lentivirus vectors (Figure 3A). Earlier, it was shown that lentivirus vectors efficiently introduced exogenous genes into the Sertoli cells of adult testes without transduction of SSCs protected by the blood-testis

barrier (Ikawa et al., 2002). We used this system to explore the effect of FGF2 on SSCs. In the first set of experiments, we prepared a lentivirus vector expressing FGF2. Empty vector was used as a control. Virus particles were microinjected into the seminiferous tubules of adult mice. One month after microinjection, testis cells were dissociated into single cells via enzymatic digestion. Real-time PCR analysis of the recovered cells showed that the level of expression of mRNA encoding *Fgf2* was ~2.1-fold greater than that in control (Figure 3B), but immunohistochemical analysis of testis slices showed that numbers of undifferentiated and differentiating spermatogonia per tubule did not change upon *Fgf2* overexpression (OE; Figure 3C). To enumerate SSCs, we transplanted equal numbers of cells into seminiferous tubules of W mice. Analysis of recipient mice showed that *Fgf2* OE did not affect the number of colonies produced (Figures 3D and 3E). Testis cells from *Fgf2* treated or control mice produced 0.6 ± 0.1 and 0.9 ± 0.2 colonies per 10^5 transplanted cells (n = 24), and the difference was not significant.

In the second set of experiments, we injected *Fgf2* knockdown (KD) lentivirus vectors. We collected testes 1 month after microinjection of virus particles into seminiferous tubules of mice. Real-time PCR analysis of dissociated testis cells confirmed that the *Fgf2* mRNA expression level in testes injected with the *Fgf2* KD vector was ~81.8% that of the control (n = 5; Figure 3B). Immunohistochemical staining showed that the number of $CDH1^+$ spermatogonia in early (I–V) and middle (VI–VIII) stages of spermatogenesis increased significantly by *Fgf2* KD (Figure 3C). However, the numbers of $GFRA1^+$ or KIT^+ spermatogonia per tubule did not show significant differences. Transplantation experiments revealed significant increases in germ cell colony numbers after *Fgf2* KD (Figures 3D and 3E). The numbers of colonies generated by testis cells injected with *Fgf2* KD or the control vectors were 4.1 ± 0.5 and 1.4 ± 0.3 per 10^5 transplanted cells (n = 18), suggesting that *Fgf2* depletion increased the frequency of SSCs in the transplanted cell suspension.

As an increase in spermatogonial number was likely attributable to changes in cytokine expression, we next examined whether FGF2 synthesis influenced the GDNF expression level. Western blotting showed that the GDNF level was significantly increased in testes treated with the *Fgf2* KD vector (Figure 3F). As no change was evident at the mRNA level (Figure 3B), the data suggested that the GDNF protein level is regulated post-transcriptionally by FGF2. In contrast, GDNF protein did not change significantly under the *Fgf2* OE condition. Similar results were obtained when we used W mice that do not have endogenous spermatogenesis (Figures S3A–S3C). These results suggest that FGF2 expression in Sertoli cells influences GDNF levels.

Proliferation of Spermatogonial Clusters upon FGF2 Stimulation

Although our work with *Ret* mutant mice suggested that SSCs might survive without GDNF, it was still possible that signal transduction pathways that do not depend on Y1062 phosphorylation might have promoted survival of undifferentiated spermatogonia. In addition, a potential involvement of RET-independent GDNF signaling via NCAM could not be excluded (Paratcha et al., 2003). Therefore, we directly examined whether SSCs could remain viable without GDNF in vitro. Testes cells from 5- to 10-day-old mice were cultured on gelatin-coated plates in different cytokine environments. Pup testes are relatively enriched for SSCs because of a lack of haploid germ cells and have been used to derive GS cell cultures (Shinohara et al., 2001; Takashima et al., 2013). Germ cells were transferred to LN-coated plates on the day after first plating to remove somatic cells. Testis cells cultured in the absence of any cytokine underwent apoptosis within 3 days, but the addition of either GDNF or FGF2 induced spermatogonial proliferation. Spermatogonia cultured with FGF2 (F-SPG) consisted predominantly of flat 2D colonies, but most spermatogonia cultured with GDNF (G-SPG) consisted of clumps; the colonies were thus 3D (Figure 4A). In F-SPG cultures, $CDH1^+$ cells increased in number upon culture in >5 ng/ml FGF2 (Figure 4B). Phalloidin stained the actin of G-SPG colonies more strongly (Figure 4C).

Typically, fibroblasts disappeared after three to four passages on LN-coated plates, and F-SPG and G-SPG proliferated steadily. Although the growth of F-SPG was relatively slow compared with that of G-SPG, both cell types were passaged at a ratio of ~1:3 every 6 days during the stable growth phase (Figure 4D). In contrast, when both FGF2 and GDNF were used to initiate GS cell cultures, they proliferated more actively, and cells were passaged every 5 to 6 days at a ratio of 1:3 to 1:5, suggesting a synergistic action between GDNF and FGF2. G-SPG adhered less efficiently to LN than did F-SPG cells (Figure 4E), possibly attributable to the difference in colony morphology. Moreover, GDNF, but not FGF2, could rescue apoptosis when testis cells were cultured on poly L-lysine (PLL)-coated plates, suggesting that integrin-mediated signaling and GDNF acted in an additive manner to support SSC survival (Figures 4F and 4G).

To examine the phenotypes of the cultured cells, we analyzed the expression levels of cell surface makers via flow cytometry (Figure 4H). Both cell types expressed ITGA6, ITGB1, CD9, EPCAM, and CDH1 (all are markers of SSCs; Kanatsu-Shinohara and Shinohara, 2013). However, KIT was significantly upregulated only in F-SPG. Although a previous study showed that GFRA1 expression increased upon GDNF stimulation of cultured spermatogonia (Oatley et al., 2006), we were unable to detect any

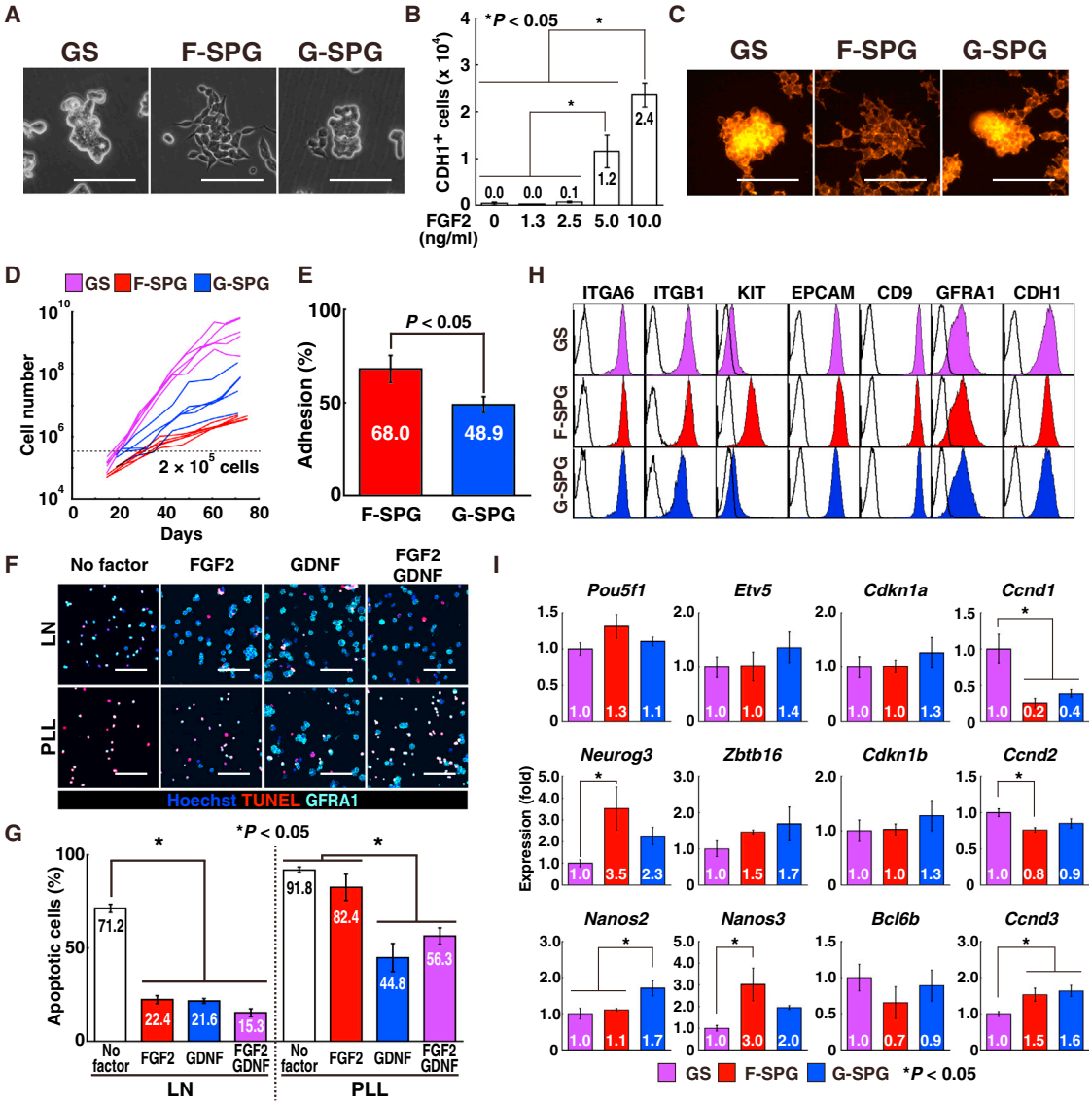


Figure 4. Proliferation of Spermatogonia in the Absence of GDNF

(A) The appearance of germ cell colonies in the presence of different cytokines. GS cells, spermatogonia culture derived by FGF2 and GDNF; F-SPG, spermatogonia culture derived by FGF2; G-SPG, spermatogonia culture derived by GDNF.

(B) Dose dependency of F-SPG proliferation. Testis cells (2×10^5 cells/3.8 cm^2) were cultured on LN for 2 weeks after gelatin selection, and the numbers of $CDH1^+$ cells were determined via flow cytometry (n = 3 experiments). Controls omitted the primary antibody staining.

(C) Phalloidin staining.

(D) Growth curve of cultured cells (FGF2, 10 ng/ml; GDNF, 10 ng/ml).

(E) LN binding ability of cultured cells (n = 12 experiments). Cells (2×10^5 cells/3.8 cm^2) were incubated on LN for 30 min.

(F) Apoptosis of pup testis cells under different culture conditions. Testis cells (2×10^5 cells/3.8 cm^2) were cultured for 4 days and stained with an anti-GFRA1 antibody. Apoptotic cells were visualized by terminal deoxynucleotidyl transferase dUTP nick end labeling staining.

(G) Quantification of $GFRA1^+$ cells undergoing apoptosis on LN- or PLL-coated plates (n = 3 experiments). At least 110 $GFRA1^+$ cells were counted in each experiment. Although GDNF suppressed apoptosis of cells cultured on both PLL and LN, FGF2 did not suppress apoptosis of cells cultured on PLL.

(H) Flow cytometric analysis. Black lines indicate isotype controls.

(I) Real-time PCR analysis. All values were normalized to the expression levels of the relevant gene in GS cells (n = 9 experiments).

Scale bars represent 100 μm (A, C, and F). Results are means ± SEM. See also Tables S1, S3, and S4.

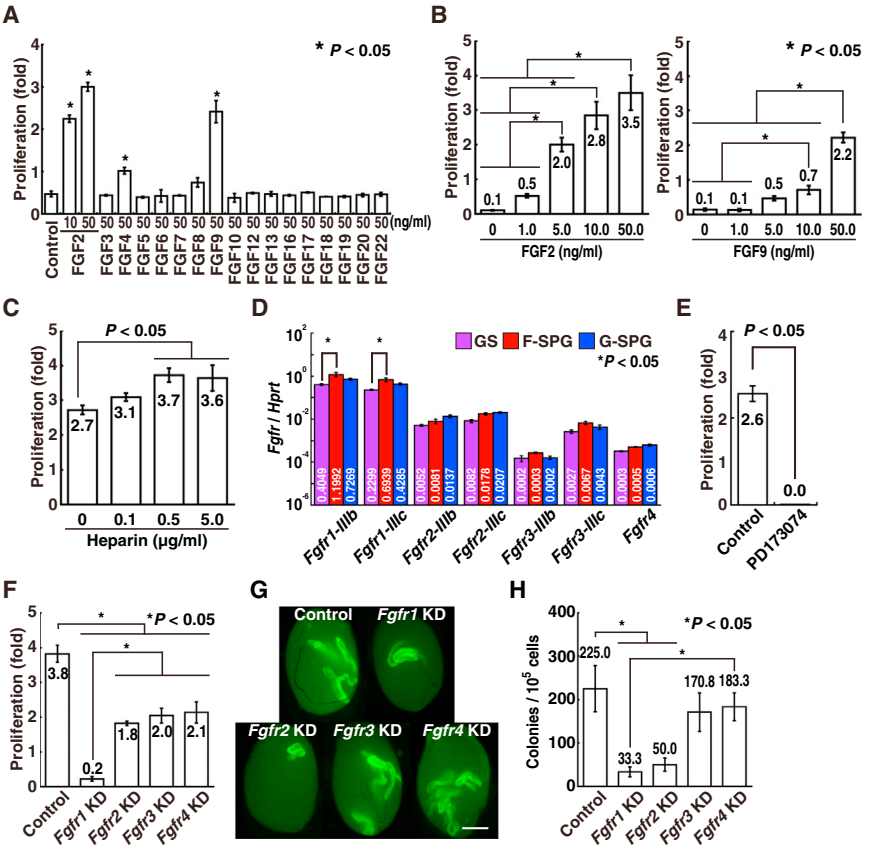


Figure 5. Stimulation of F-SPG by FGFR1

(A) Stimulation of F-SPG cell proliferation by FGF family molecules (n = 3 experiments). Cells (2 × 10^5 cells per 3.8 cm^2) were cultured for 6 days on LN.

(B) Dose dependency of FGF2 and FGF9 stimulation (n = 6 experiments). Cells (1.5 × 10^5 cells per 3.8 cm^2) were cultured for 6 days on LN.

(C) Stimulatory effect of heparin in FGF2 culture (n = 6 experiments). Cells (1.5 × 10^5 cells per 3.8 cm^2) were cultured for 6 days on LN.

(D) Real-time PCR analysis of the levels of *Fgfr* expression (n = 9 experiments). Transcript levels were normalized to those of *Hprt*.

(E) Suppression of F-SPG proliferation by PD173074 (n = 4 experiments). Cells (2 × 10^5 cells per 3.8 cm^2) were cultured for 6 days on LN with 0.2 μM PD173074.

(F) Suppression of F-SPG proliferation by *Fgfr1* KD (n = 3 experiments). Cells were transduced with the indicated KD vectors and plated on LN (1.5 × 10^5 cells per 3.8 cm^2) 4 days after transfection. Cells were recovered 9 days after plating.

(G) Macroscopic appearance of recipient testes that were transplanted with F-SPG 2 days after transduction with the indicated vectors.

(H) Colony counts (n = 12 testes).

Results are means ± SEM. See also Figure S4 and Tables S1, S2, and S4.

difference in GFRA1 expression levels between the two types of cells. Real-time PCR analysis revealed that *Nanos2* was significantly upregulated in G-SPG, whereas *Nanos3* and *Neurog3* were both strongly expressed in F-SPG (Figure 4I). The *Ccnd1* levels of both F-SPG and G-SPG were lower than that of GS cells, but the *Ccnd3* levels were higher, suggesting changes in the self-renewal and differentiation patterns.

Stimulation of SSC Self-Renewal by FGFR1

FGF2 is expressed by Sertoli cells (Mullaney and Skinner, 1992). However, at least 23 forms of the FGF family molecules exist (Itoh and Ornitz, 2011), and we thus tested the effects of various FGF family members on proliferation of F-SPG (Figure 5A). Cell proliferation was efficiently stimulated by FGF4, and more strongly by FGF9. However, FGF9 was not as effective as FGF2 (Figure 5B). Adding heparin increased cell proliferation in FGF2 culture (Figure 5C). Real-time PCR revealed that all tested cells expressed predominantly *Fgfr1-IIIb* and *Fgfr1-IIIc* (Figure 5D). The gene expression levels were greatest in F-SPG. The addition of PD173074, an inhibitor of both FGFR1 and FGFR3, suppressed F-SPG proliferation (Figure 5E). To examine the impact of each receptor on proliferation, we transfected F-SPGs with lentiviruses expressing shRNAs against *Fgfr1* to *Fgfr4* (Figure S4). Although depletion of any of these genes inhibited proliferation of F-SPG, *Fgfr1* depletion afforded the most significant effect: only ~5% of the initially plated cells were recovered (Figure 5F). We also carried out transplantation experiments. F-SPG were transplanted 2 days after transfection with shRNA against *Fgfr1* to *Fgfr4*. Analysis of recipient testes showed that depletion of *Fgfr1* or *Fgfr2* significantly decreased the number of colonies (Figures 5G and 5H). These results suggested that FGFR1 and FGFR2 are expressed on SSCs and that *Fgfr1* plays a dominant role in F-SPG proliferation.

MAP2K1/2-Independent SSC Self-Renewal in F-SPG

To examine the mechanism of FGF2-induced self-renewal, we first analyzed the effect of LN on the phosphorylation of RET, a critical component of the GDNF receptor (Sariola and Saarma, 2003). The *Ret* mutation at Y1062 inhibits SSC self-renewal and triggers hypospermatogenesis (Jijiwa et al., 2008), and phosphorylation of RET Y1015 activates

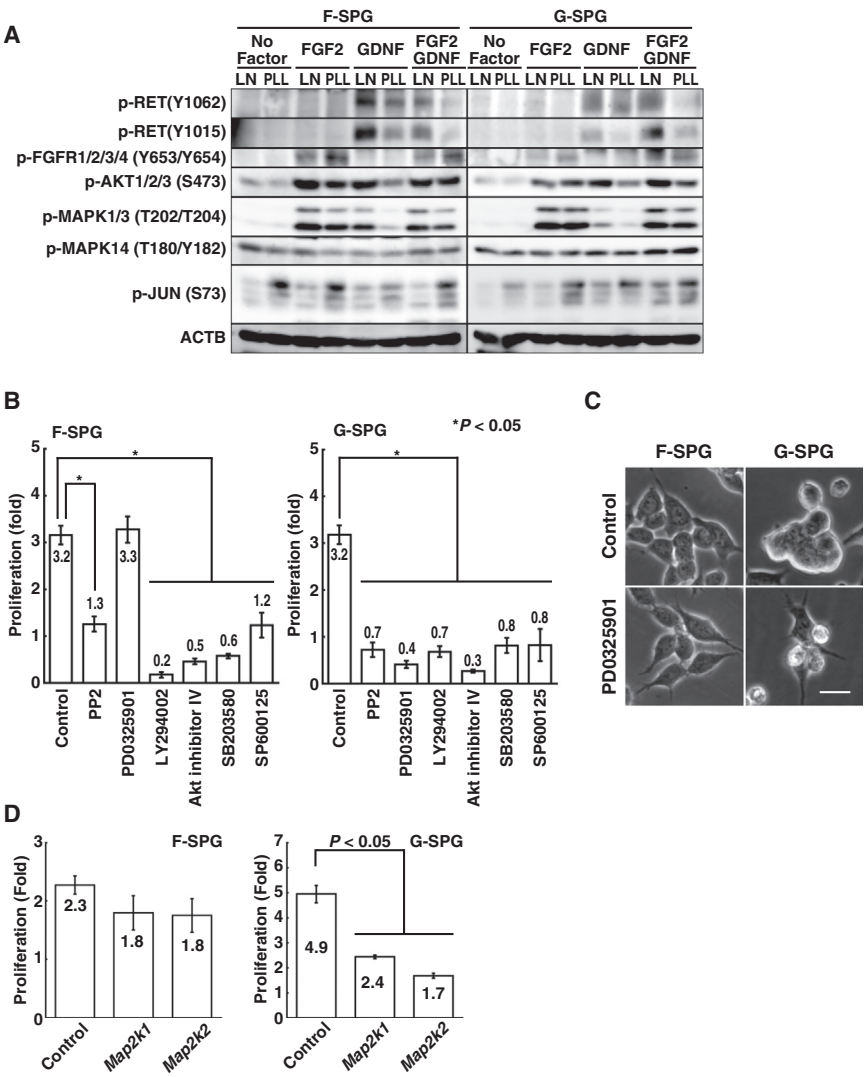


Figure 6. Signaling Mechanisms Involved in FGF2 or GDNF Stimulation

(A) Western blotting of F-SPG or G-SPG stimulated under different conditions. F-SPG and G-SPG (5×10^6 cells per 55 cm^2) were plated on LN and cultured for 3 days without cytokines. Cells were restimulated with FGF2 (10 ng/ml) or GDNF (10 ng/ml) for 10 min before sample collection. Cells that had not been treated with cytokines were used as control (No factor).

(B) Effects of chemical inhibitors on F-SPG or G-SPG proliferation (n = 8–26 experiments). Cells (2×10^5 cells per 3.8 cm^2) were cultured for 6 days.

(C) Appearance of F-SPG or G-SPG treated with PD0325901.

(D) Suppression of F-SPG proliferation by depletion of the indicated genes (n = 6–12 experiments). Cells (1.5×10^5 cells per 3.8 cm^2) were cultured for 6 days on LN after transfection with the indicated vectors.

The scale bar represents 20 μm (C). Results are means ± SEM. See also Figure S5 and Tables S1–S4.

phospholipase C (Fukuda et al., 2002). GDNF induced phosphorylation of RET Y1015 and Y1062, but the phosphorylation pattern did not differ between F-SPG and G-SPG (Figure 6A). Phosphorylation of both Y1015 and Y1062 was enhanced when cells were cultured on LN. However, we detected no influence of FGF2 on RET phosphorylation levels. Phosphorylation of AKT and MAPK1/3 was mildly enhanced upon culture on LN. AKT phosphorylation levels were similar in F-SPG and G-SPG when they were stimulated with FGF2 and GDNF, respectively. However, although MAPK1/3 phosphorylation was evident by FGF2, GDNF increased phosphorylation levels only modestly, suggesting that FGF2 plays a more important role in MAPK1/3 phosphorylation. Interestingly, although we did not observe significant changes in MAPK14 phosphorylation levels, culture on PLL induced apparent increases in the extent of JUN phosphorylation, suggesting that JUN may plays a role in induction of apoptosis caused by a lack of integrin-mediated signaling.

As AKT or MAPK1/3 phosphorylation levels were upregulated in F-SPG or G-SPG, we next used chemical inhibitors to clarify the roles played by these molecules in the context of self-renewal (Figure 6B). PP2 (a Src-family kinase inhibitor), LY294002 (a phosphoinositide 3-kinase (PIK3) inhibitor), and the Akt inhibitor IV significantly reduced the recovery levels of both F-SPG and G-SPG, suggesting that Src-family molecules and the PIK3-AKT pathway are both involved in self-renewal. We found no apparent differences between the actions of SB203580 (a MAPK14 inhibitor) and SP600125 (a MAPK8 inhibitor), both of which effectively suppressed GS cell proliferation in our previous study (Morimoto et al., 2013). Similar results were obtained when we used different chemical inhibitors for each signaling pathway (Figure S5A). In contrast, PD0325901

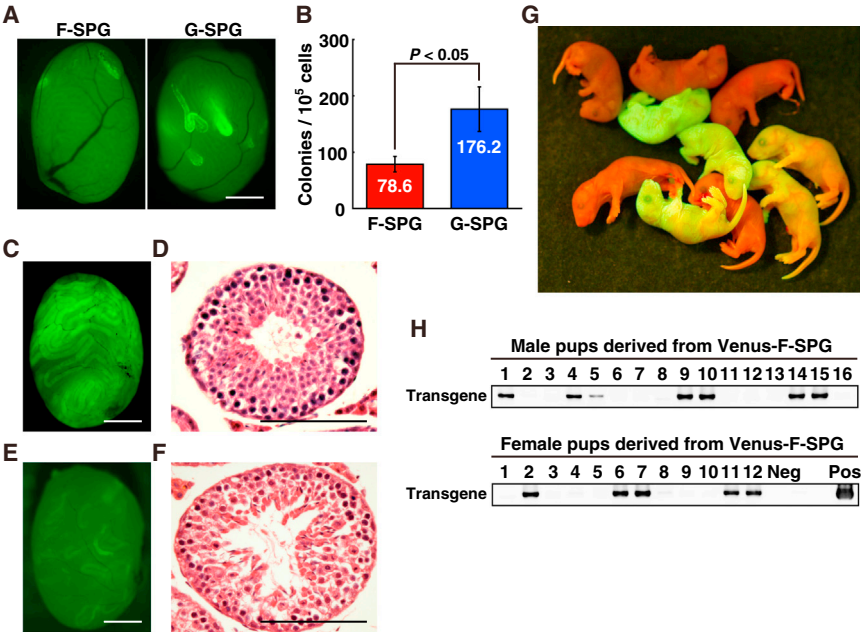


Figure 7. Germline Transmission of F-SPG

(A) Macroscopic appearance of recipient testes.

(B) Colony counts (n = 21 testes).

(C and D) Macroscopic (C) and histological (D) appearance of a recipient testis transplanted with freeze-thawed G-SPG.

(E and F) Macroscopic (E) and histological (F) appearance of a recipient testis transplanted with Venus-expressing F-SPG.

(G) Offspring born from G-SPG transplantation and microinsemination.

(H) PCR data revealing integration of the transgene in offspring born from F-SPG.

Scale bars represent 1 mm (A, C, and E) and 100 μm (D and F). Results are means ± SEM. See also Tables S4 and S5.

(a MAP2K1/2 inhibitor) differentially influenced cell recovery (Figures 6B and 6C). Although this inhibitor did not significantly affect F-SPG proliferation, the number of G-SPG declined significantly in the presence of PD0325901, suggesting that MAP2K1/2 was necessary for both the self-renewal and survival of G-SPG. This was true despite the fact that the phosphorylation level of MAPK1/3 in G-SPG was relatively low. Results of PD0325901 were confirmed by depletion of target gene expression by shRNA (Figures 6D, S5B, and S5C). These results suggest that MAP2K1/2 is dispensable for F-SPG, which may depend more strongly on AKT action.

Germline Transmission of Cultured Cells

Strong KIT expression in F-SPG suggested that they were progenitors. Therefore, we performed germ cell transplantation to address this question. F-SPG and G-SPG derived from green mice were maintained in vitro for 139 days. During passage, some cells were transplanted into seminiferous tubules to measure increases in SSC number. On long-term culture, G-SPG proliferated more actively than did F-SPG, expanding 4.5 × 10^8-fold, whereas F-SPG expanded only 7.6 × 10^3-fold over 139 days. In contrast, FGF2 and GDNF acted synergistically to expand GS cell numbers 8.2 × 10^6-fold over 65 days.

Examination of recipient mice under UV light revealed that both cultures contained SSCs (Figure 7A). However, colony counts showed that G-SPG produced significantly more colonies than did F-SPG (Figure 7B), suggesting that F-SPG contain SSCs albeit with a lower frequency. Overall, a 2.3 × 10^5-fold expansion of SSCs was observed over 139 days of G-SPG culture, whereas the figure for in F-SPG culture was 4.7 × 10^3-fold over the same period. The doubling times of F-SPG and G-SPG were 11.4 and 7.4 days, respectively, during stable growth. The doubling time of G-SPG was comparable to the estimated doubling time of SSCs during regeneration in vivo (~7.9 days; Nagano, 2003), suggesting that SSCs undergo self-renewal more efficiently when supplemented with GDNF.

Finally, we explored whether G-SPG and F-SPG can produce offspring. In the first set of experiments, G-SPGs from a green mouse that had been cultured for 88 days were transplanted into seminiferous tubules of W mice after freeze thawing. In the second set of experiments, we transfected F-SPG from a WT mouse with a lentivirus vector expressing Venus at 174 days after culture initiation, and transfected cells were microinjected into seminiferous tubules of W mice on the next day. Recipient testes were recovered 2 and 4 months after transplantation, respectively, for G-SPG and F-SPG experiments (Figures 7C–7F). Donor-derived germ cells were collected via mechanical dissociation of seminiferous tubules exhibiting donor-cell derived fluorescence. Spermatozoa were microinjected into oocytes of C57BL/6 × DBA/2 F1 (BDF1) mice, and two-cell embryos were transferred into the uteri of pseudopregnant mice (Table S5). Offspring were obtained from both G-SPG and F-SPG. Donor cell origin of G-SPG was confirmed by EGFP fluorescence under UV light (Figure 7G). On the other hand, PCR analysis of transgene expression showed that 12 of 28 offspring from F-SPG contained the transgene (Figure 7H) and weakly exhibited Venus fluorescence (data not shown). These results

indicate that both G-SPG and F-SPG undergo germline transmission.

DISCUSSION

Both GDNF and FGF2 are expressed by Sertoli cells, but very little is known about the roles played by these materials in vivo or the spatial relationship among such cells and the SSCs of seminiferous tubules. In the present study, we used a *Ret* mutant mouse strain to explore whether SSCs survived in the absence of GDNF signaling. Although spermatogonial transplantation is usually the best approach to testing for the presence of SSCs, we reasoned that analysis of the outcomes of germ cell transplantation from *Ret* or *Gfra1* mutant mice might not be useful in the present context. As GDNF is apparently involved in spermatogonial proliferation, it was possible that lack of such signaling would limit colony expansion. In addition, GDNF has been implicated in migration of SSCs to their niches (Kanatsu-Shinohara et al., 2012). Therefore, we decided to analyze the in vivo development of mutant testes by transplanting testis fragments of *Ret* mutant mice into surrogate animals.

We clearly showed that undifferentiated spermatogonia survived despite the absence of Y1062 phosphorylation, which is thought to be required for SSC self-renewal (Jain et al., 2004; Jijiwa et al., 2008). Although the frequency of germ cell clusters obtained was low, seven of eight transplanted fragments contained areas of mutant tubules with $CDH1^+$ cells. Such results were unexpected because clusters of this type have not been reported in previous analyses of *Gdnf*/*Ret*/*Gfra1* KO mice. The failure to detect germ cells in previous studies might be attributable to the lack of surrounding host Leydig cells in subcutaneous tissue (Naughton et al., 2006); such cells are thought to synthesize niche factors (Oatley et al., 2009). In this context, transplantation of testis fragments to busulfan-treated testes, as in the present study, may have afforded a better proliferative environment in that Leydig cells were present. Thus, our finding raise the possibility that a subset of A_s spermatogonia survive and proliferate in the absence of GDNF signaling.

It is intriguing that all surviving germ cells in *Ret* mutant mice expressed GFRA1. It is possible that GFRA1 was upregulated to compensate for the reduction of GDNF signaling. Because of the controversy on GFRA1 expression on SSCs, we performed FACS experiments to explore the relationship between GFRA1 expression and SSC activity in WT mice. The FACS studies suggested that most SSCs were $GFRA1^+$ cells. Although GFRA1 expression by SSCs was not noted in several previous studies (Buageaw et al., 2005; Ebata et al., 2005; Grisanti et al., 2009), the cited works were performed using MACS, and GFRA1 expression levels were not quantitated. We are currently unable to definitively explain the discrepancy between our results and those of cited studies, but differences in the enzymatic digestion protocols used to obtain single-cell suspensions may have affected the expression levels of GFRA1. In previous studies, different protocols were employed to obtain testis cell single-cell suspensions. We used only collagenase type II to this end, whereas two- or three-step digestion protocols were earlier used. As GFRA1 is a GPI-anchored protein that can be solubilized (Sariola and Saarma, 2003), extensive enzymatic digestion may influence the expression levels thereof. In addition, the polyclonal antibodies used in previous studies may have lacked the affinity required to allow collection of all GFRA1-expressing cells via MACS. However, although FACS revealed enrichment of SSCs in $GFRA1^+$ cells, we also found SSC activity in $GFRA1^-$ cells, and it was difficult to exclude completely that GFRA1 is not expressed by some SSCs.

As FGF2 exerts beneficial effects on cultured spermatogonia, we explored the impact of FGF2 in vivo. Interestingly, FGF2 did not act in a manner similar to GDNF. Because of the importance of FGF2 in term of GS cell proliferation, we initially hypothesized that *Fgf2* OE would increase SSC numbers in a manner similar to that noted upon induction of *Gdnf* OE (Yomogida et al., 2003) and that *Fgf2* KD would reduce SSC numbers. However, the number of germ cell colonies generated upon *Fgf2* OE was not greatly different from that of the control, whereas *Fgf2* KD actually increased the number of germ cell colonies. As colony number thus increased in an *Fgf2* KD environment, it appears that the proportion of SSCs supported by FGF2 may be relatively small compared with GDNF. It also suggests that FGF2 act more strongly on progenitors. Expression of FGF2 and GDNF may not be totally irrelevant because *Fgf2* KD triggered increased GDNF expression in the testis. Thus, these experiments showed differences in FGF2 and GDNF function and complexity of GDNF expression in vivo, but the data did not allow us to understand how germ cells survive in *Ret* mutant mice.

We directly analyzed the impact of FGF2 in vitro. Derivation of F-SPG indicates that at least some SSCs undergo self-renewal in the complete absence of GDNF. We also found that FGF9, which was previously used in rat GS cell culture (Kanatsu-Shinohara et al., 2011), can also induce F-SPG proliferation. FGF2 and FGF9 commonly bind to FGFR2 and FGFR3 (Itoh and Ornitz, 2011). Because both FGFR2 and FGFR3 are shown to be involved in spermatogonia proliferation, we expected that either FGFR2 or FGFR3 would be involved in F-SPG proliferation. However, depletion of *Fgfr1* showed the most significant effect. We speculate

that this marked effect of *Fgfr1* KD could reflect the relatively higher *Fgfr1* expression, given that depletion of not only *Fgfr1* but also *Fgfr2* reduced germ cell colony numbers after transplantation. Although the exact relationship of ligand-receptor interaction in vivo may be difficult to study due to availability of proteoglycans and complexity of FGF-FGFR signaling, our derivation of F-SPG suggests that GFRA1$^+$ cells in *Ret* mutant mice might have survived by FGF molecules secreted from Sertoli cells.

Although both GDNF and FGF2 similarly promoted proliferation, at least five differences were noted. First, GDNF was much weaker at inducing MAPK1/3 phosphorylation. Second, FGF2 stimulated formation of flat colonies, whereas GDNF induced colony clumping, suggesting that FGF2 and GDNF play distinct roles in cytoskeletal organization. Third, FGF2 could not rescue apoptosis of cells growing on PLL; such apoptosis was suppressed by GDNF. Fourth, self-renewal caused by FGF2 could not be suppressed via MAP2K1/2 inhibition, but such treatment did in fact abrogate G-SPG proliferation. Finally, SSC activity was significantly higher in G-SPG than F-SPG. These results show that SSCs exhibit at least two modes of self-renewal.

To understand how each cytokine regulates SSC self-renewal in vivo is a next important goal. Our results suggest that SSC self-renewal is not simply regulated by elevated GDNF expression. Given that MAPK1/3 activation was evident in GFRA1$^+$ cells of stages I–III and VII–VIII when GDNF expression was found in Sertoli cells of stages II–VI in WT testes (Grasso et al., 2012; Hasegawa et al., 2013; Sato et al., 2011), some GFRA1$^+$ cells appear to undergo MAPK1/3 phosphorylation under conditions where GDNF is apparently not expressed, suggesting that FGF2 is involved in the activities of cells of stages I and VII–VIII. Although it is possible that F-SPG and G-SPG may have different cellular origins, we were not able to address this issue in this manuscript. However, because of the relatively similar cytokine response (Figure 6A), we rather think that all SSCs are equally competent in terms of responding to GDNF or FGF2 and that such cells change their mode of self-renewal and phenotype depending on the availability of GDNF or FGF2. Further studies are required to explore the origins and relationships among these two cell populations in vivo.

We found that GDNF was not a prerequisite for SSC survival or self-renewal, challenging the traditional belief that GDNF is indispensable for SSC viability. Moreover, our work raises questions about the mechanism of self-renewal and the heterogeneity of SSC populations: How do GDNF and FGF signaling differ in the context of self-renewal? Does an SSC hierarchy exist? Our results provide critical insights into SSC biology and the regulation of spermatogenesis.

EXPERIMENTAL PROCEDURES

Animals and Cell Culture

Ret mutant mice have been described previously (Jijiwa et al., 2008). GS cells were derived from the transgenic mouse line C57BL/6 Tg14(act-EGFP)OsbY01 (gift from Dr. M. Okabe, Osaka University), which were backcrossed into the DBA/2 background for at least seven generations. We also used ROSA mice (Jackson Laboratory) for FACS experiments. Where indicated, 6-day-old pups of the DBA/2 background were used to initiate testis cell cultures. GS cell culture conditions have been described previously (Kanatsu-Shinohara et al., 2003). GS cells were maintained on dishes coated with 20 μg/ml LN (BD Biosciences).

For proliferation assays, 1.5×10^5 cells were plated on LN-coated 12-well plates (20 μg/ml; BD Biosciences). Heparin sodium salt (Sigma), PP2 (10 μM), PD173074 (0.2 μM), LY294002 (33 μM), Akt inhibitor IV (80 nM), SB203580 (60 μM; Calbiochem), BIRB 796 (10 μM), PP1 (2 μM), MK-2206 (3 μM), Jnk inhibitor IX (3 μM), PD0325901 (2 μM), and SP600125 (40 μM; Selleck Chemicals) were added at the time of plating. All cytokines used in the present study are listed in Table S1. For adhesion assays, the plates were washed with PBS, and the cells recovered in Cell Dissociation Buffer (CDB; Invitrogen); 2×10^5 cells were incubated in wells of 12-well plates coated with LN for 30 min. Cells were recovered with the aid of trypsin. Where indicated, we also coated dishes with PLL (0.0017%; Sigma).

Transplantation

Ret mutant mice carrying *Egfp* gene were produced by crossing *Ret* mutant and green mice. Individual testes were dissected into two fragments using fine forceps. For transplantation, 8- to 12-week-old KSN nude mice were injected intraperitoneally with busulfan (44 mg/kg) at 4 weeks of age. Within 3 to 4 days, these animals received bone marrow transplantations to avoid bone marrow failure. At least 4 weeks after busulfan treatment, the mice were anesthetized and a small cut was made in the tunica albuginea of each animal using fine forceps. A single graft was inserted 3- to 4-mm deep into the testicular parenchyma.

For germ cell transplantation, we used W mice (Japan SLC) that are congenitally infertile due to mutations in *Kit* (Geissler et al., 1988). Cells were transplanted into W mice when the recipients were 6–10 weeks of age. Approximately 4-μl amounts of cell suspension were injected through the efferent duct (Ogawa et al., 1997). Each injection filled 75%–85% of all seminiferous tubules. The Institutional Animal Care and Use Committee of Kyoto University approved all of our animal experimentation protocols.

Statistical Analyses

Significant differences between means for single comparisons were determined by Student's t tests. Multiple comparison analyses were carried out using ANOVA followed by Tukey's Honest Significant Difference test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and five tables and can be found

with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.01.010.

ACKNOWLEDGMENTS

We thank Ms. Y. Ogata for technical assistance. Financial support for this research was provided by The Uehara Memorial Foundation, and The Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Japan Science and Technology Agency (PRESTO).

Received: August 5, 2014
Revised: January 12, 2015
Accepted: January 13, 2015
Published: February 12, 2015

REFERENCES

Bellus, G.A., Hefferon, T.W., Ortiz de Luna, R.I., Hecht, J.T., Horton, W.A., Machado, M., Kaitila, I., McIntosh, I., and Francomano, C.A. (1995). Achondroplasia is defined by recurrent G380R mutations of FGFR3. Am. J. Hum. Genet. *56*, 368–373.

Brinster, R.L., and Zimmermann, J.W. (1994). Spermatogenesis following male germ-cell transplantation. Proc. Natl. Acad. Sci. USA *91*, 11298–11302.

Buageaw, A., Sukhwani, M., Ben-Yehudah, A., Ehmcke, J., Rawe, V.Y., Pholpramool, C., Orwig, K.E., and Schlatt, S. (2005). GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol. Reprod. *73*, 1011–1016.

Chiarini-Garcia, H., Hornick, J.R., Griswold, M.D., and Russell, L.D. (2001). Distribution of type A spermatogonia in the mouse is not random. Biol. Reprod. *65*, 1179–1185.

de Rooij, D.G., and Russell, L.D. (2000). All you wanted to know about spermatogonia but were afraid to ask. J. Androl. *21*, 776–798.

Ebata, K.T., Zhang, X., and Nagano, M.C. (2005). Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. Mol. Reprod. Dev. *72*, 171–181.

Fukuda, T., Kiuchi, K., and Takahashi, M. (2002). Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. J. Biol. Chem. *277*, 19114–19121.

Geissler, E.N., Ryan, M.A., and Housman, D.E. (1988). The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. Cell *55*, 185–192.

Goriely, A., McVean, G.A., van Pelt, A.M., O'Rourke, A.W., Wall, S.A., de Rooij, D.G., and Wilkie, A.O. (2005). Gain-of-function amino acid substitutions drive positive selection of FGFR2 mutations in human spermatogonia. Proc. Natl. Acad. Sci. USA *102*, 6051–6056.

Grasso, M., Fuso, A., Dovere, L., de Rooij, D.G., Stefanini, M., Boitani, C., and Vicini, E. (2012). Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction *143*, 325–332.

Grisanti, L., Falciatori, I., Grasso, M., Dovere, L., Fera, S., Muciaccia, B., Fuso, A., Berno, V., Boitani, C., Stefanini, M., and Vicini, E. (2009). Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells *27*, 3043–3052.

Hasegawa, K., Namekawa, S.H., and Saga, Y. (2013). MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. Stem Cells *31*, 2517–2527.

Ikawa, M., Tergaonkar, V., Ogura, A., Ogonuki, N., Inoue, K., and Verma, I.M. (2002). Restoration of spermatogenesis by lentiviral gene transfer: offspring from infertile mice. Proc. Natl. Acad. Sci. USA *99*, 7524–7529.

Ishii, K., Kanatsu-Shinohara, M., Toyokuni, S., and Shinohara, T. (2012). FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. Development *139*, 1734–1743.

Itoh, N., and Ornitz, D.M. (2011). Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease. J. Biochem. *149*, 121–130.

Jain, S., Naughton, C.K., Yang, M., Strickland, A., Vij, K., Encinas, M., Golden, J., Gupta, A., Heuckeroth, R., Johnson, E.M., Jr., and Milbrandt, J. (2004). Mice expressing a dominant-negative Ret mutation phenocopy human Hirschsprung disease and delineate a direct role of Ret in spermatogenesis. Development *131*, 5503–5513.

Jijiwa, M., Kawai, K., Fukihara, J., Nakamura, A., Hasegawa, M., Suzuki, C., Sato, T., Enomoto, A., Asai, N., Murakumo, Y., and Takahashi, M. (2008). GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. Genes Cells *13*, 365–374.

Kanatsu-Shinohara, M., and Shinohara, T. (2013). Spermatogonial stem cell self-renewal and development. Annu. Rev. Cell Dev. Biol. *29*, 163–187.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., and Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol. Reprod. *69*, 612–616.

Kanatsu-Shinohara, M., Ikawa, M., Takehashi, M., Ogonuki, N., Miki, H., Inoue, K., Kazuki, Y., Lee, J., Toyokuni, S., Oshimura, M., et al. (2006). Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. Proc. Natl. Acad. Sci. USA *103*, 8018–8023.

Kanatsu-Shinohara, M., Kato-Itoh, M., Ikawa, M., Takehashi, M., Sanbo, M., Morioka, Y., Tanaka, T., Morimoto, H., Hirabayashi, M., and Shinohara, T. (2011). Homologous recombination in rat germline stem cells. Biol. Reprod. *85*, 208–217.

Kanatsu-Shinohara, M., Inoue, K., Takashima, S., Takehashi, M., Ogonuki, N., Morimoto, H., Nagasawa, T., Ogura, A., and Shinohara, T. (2012). Reconstitution of mouse spermatogonial stem cell niches in culture. Cell Stem Cell *11*, 567–578.

Lee, J., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Kimura, T., Nakano, T., Ogura, A., and Shinohara, T. (2007). Akt mediates self-renewal division of mouse spermatogonial stem cells. Development *134*, 1853–1859.

Li, L., and Xie, T. (2005). Stem cell niche: structure and function. Annu. Rev. Cell Dev. Biol. *21*, 605–631.

Meistrich, M.L., and van Beek, M.E.A.B. (1993). Spermatogonial stem cells. In Cell and Molecular Biology of the Testis, C. Desjardins and L.L. Ewing, eds. (Oxford University Press), pp. 266–295.

Meng, X., Lindahl, M., Hyvönen, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science *287*, 1489–1493.

Morimoto, H., Iwata, K., Ogonuki, N., Inoue, K., Ogura, A., Kanatsu-Shinohara, M., Morimoto, T., Yabe-Nishimura, C., and Shinohara, T. (2013). ROS are required for mouse spermatogonial stem cell self-renewal. Cell Stem Cell *12*, 774–786.

Mullaney, B.P., and Skinner, M.K. (1992). Basic fibroblast growth factor (bFGF) gene expression and protein production during pubertal development of the seminiferous tubule: follicle-stimulating hormone-induced Sertoli cell bFGF expression. Endocrinology *131*, 2928–2934.

Nagano, M.C. (2003). Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol. Reprod. *69*, 701–707.

Naughton, C.K., Jain, S., Strickland, A.M., Gupta, A., and Milbrandt, J. (2006). Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol. Reprod. *74*, 314–321.

Oatley, J.M., Avarbock, M.R., Telaranta, A.I., Fearon, D.T., and Brinster, R.L. (2006). Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc. Natl. Acad. Sci. USA *103*, 9524–9529.

Oatley, J.M., Oatley, M.J., Avarbock, M.R., Tobias, J.W., and Brinster, R.L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development *136*, 1191–1199.

Ogawa, T., Aréchaga, J.M., Avarbock, M.R., and Brinster, R.L. (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. Int. J. Dev. Biol. *41*, 111–122.

Paratcha, G., Ledda, F., and Ibáñez, C.F. (2003). The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell *113*, 867–879.

Sariola, H., and Saarma, M. (2003). Novel functions and signalling pathways for GDNF. J. Cell Sci. *116*, 3855–3862.

Sato, T., Aiyama, Y., Ishii-Inagaki, M., Hara, K., Tsunekawa, N., Harikae, K., Uemura-Kamata, M., Shinomura, M., Zhu, X.B., Maeda, S., et al. (2011). Cyclical and patch-like GDNF distribution along the basal surface of Sertoli cells in mouse and hamster testes. PLoS ONE *6*, e28367.

Shinohara, T., Orwig, K.E., Avarbock, M.R., and Brinster, R.L. (2001). Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc. Natl. Acad. Sci. USA *98*, 6186–6191.

Takashima, S., Hirose, M., Ogonuki, N., Ebisuya, M., Inoue, K., Kanatsu-Shinohara, M., Tanaka, T., Nishida, E., Ogura, A., and Shinohara, T. (2013). Regulation of pluripotency in male germline stem cells by Dmrt1. Genes Dev. *27*, 1949–1958.

Tokuda, M., Kadokawa, Y., Kurahashi, H., and Marunouchi, T. (2007). CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol. Reprod. *76*, 130–141.

Yomogida, K., Yagura, Y., Tadokoro, Y., and Nishimune, Y. (2003). Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. Biol. Reprod. *69*, 1303–1307.

Yoshida, S., Sukeno, M., and Nabeshima, Y. (2007). A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science *317*, 1722–1726.